washed four times in wash buffer (lysis buffer without EGTA and Triton X-100). Recombinant Myt1 protein was eluted from the beads with 150 mM imidazole in wash buffer, frozen in liquid nitrogen, and stored at −80°C. A mock preparation was prepared in the same manner from uninfected Sf9 cells.
18. Rabbit antibodies to the COOH-terminal end of Myt1 (CNLLGMFDDATEQ) (*15*) were affinity-purified as described (*16*). Affinity-purified rabbit antibodies to mouse immunoglobulin G (Cappel) served in control experiments. All other antibodies were described previously (*9*). Endogenous Myt1 was immunoprecipitated from *Xenopus* egg extracts with anti-Myt1 as described (*4*) except that 0.5% Triton X-100 replaced NP-40.
19. An internal fragment of the Myt1 cDNA was amplified by PCR as described (*4*) except that the annealing temperature was 45°C for the first five cycles and 55°C for the remaining 30 cycles. The 5′ and 3′ primers were CGGGGTACC(C/T)T(A/G)AA(I/C)(C/T/A)TIGGIGA(T/C)(T/C)T(I/C)GG and TCCCCCGGGTGCCAI(T/G/C)II(T/A)(C/G)ICC(G/A)TT(I/C)(C/T)(G/T/C)(I/C)GG, respectively. This reaction yielded a 221–base pair fragment that was used to isolate a full-length Myt1 cDNA from a *Xenopus* oocyte library as described (*4*).
20. The conditions for in vitro kinase assays of Myt1 were as described (*4*) for Wee1 except that 0.05 % Triton X-100 was added.
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23. An interphase extract from *Xenopus* eggs was made in the presence of cycloheximide (100 μg/ml) as described (*4*). All subsequent steps were done on ice or at 4°C. The extract was diluted with two volumes of DB [20 mM Hepes (pH 7.6), 100 mM NaCl, 5 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM dithiothreitol, and protease inhibitor mix (*17*)] and centrifuged at 260,000*g* for 1 hour. The membrane fraction was resuspended in three volumes of DB containing 500 mM NaCl and recentrifuged for 30 min. This salt-washed membrane was resuspended in three volumes of DB containing 0.5 % Triton X-100, rotated for 20 min, and recentrifuged for 30 min. The resulting supernatant (the solubilized membrane fraction) was either frozen for later use or immediately subjected to immunodepletion. For this purpose, the solubilized membrane fraction was incubated with 20 μg of either anti-Myt1 or control antibodies per milliliter and bound to protein A beads as described (*18*). After this incubation, the beads were removed by centrifugation to yield Myt1-depleted or control-depleted supernatant. These depleted extracts were frozen in liquid nitrogen and stored at −80°C.
24. Cdc2 was labeled on Thr^{161} with [γ-^{32}P]ATP as described (*9*) except the reaction contained 500 μM nonradioactive ATP.
25. Immunoblotting was performed as described (*4*). Quantification was performed with a PhosphorImager (Molecular Dynamics).
26. We are grateful to D. Morgan (University of California, San Francisco) for supplying the baculovirus transfer vector, M. Solomon (Yale University) for providing Cdc2 mutant plasmids, J. Kuang (M. D. Anderson Cancer Center) for supplying MPM-2 antibodies, and B. Wold and members of the W.G.D. group for critical reading of this manuscript. Supported by grants from NIH, Lucille P. Markey Charitable Trust, and NSF. P.R.M. and T.R.C. were supported by fellowships from the Helen Hay Whitney Foundation and NIH (National Research Service Awards), respectively. P.R.M. is an associate and W.G.D. is an investigator at the Howard Hughes Medical Institute.

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Dephosphorylation of Cdk2 Thr^{160} by the Cyclin-Dependent Kinase–Interacting Phosphatase KAP in the Absence of Cyclin

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The activation of cyclin-dependent kinases (CDKs) requires the phosphorylation of a conserved threonine (Thr^{160} in Cdk2) by CDK-activating kinase (CAK). Human KAP (also called Cdi1), a CDK-associated phosphatase, was shown to dephosphorylate Thr^{160} in human Cdk2. KAP was unable to dephosphorylate Tyr^{15} and only dephosphorylated Thr^{160} in native monomeric Cdk2. The binding of cyclin A to Cdk2 inhibited the dephosphorylation of Thr^{160} by KAP but did not preclude the binding of KAP to the cyclin A–Cdk2 complex. Moreover, the dephosphorylation of Thr^{160} by KAP prevented Cdk2 kinase activity upon subsequent association with cyclin A. These results suggest that KAP binds to Cdk2 and dephosphorylates Thr^{160} when the associated cyclin subunit is degraded or dissociates.

CDKs are key regulators of cell cycle progression (*1*, *2*). Activation of Cdk2 requires cyclin binding and phosphorylation of Thr^{160} by CAK (*3*). To investigate whether the dephosphorylation of Cdk2 Thr^{160} is affected by binding to cyclin, we sought a Cdk2 substrate that could be phosphorylated by CAK to the same extent with or without the cyclin subunit. A bacterially expressed glutathione-S-transferase (GST)–Cdk2 fusion protein could be phosphorylated by CAK without binding to cyclin (Fig. 1A), although catalytic activity requires both Thr^{160} phosphorylation and cyclin A association (*4*). When the GST domain was cleaved with thrombin, the monomeric Cdk2 was phosphorylated by CAK weakly, but phosphorylation was greatly enhanced by binding to cyclin A (Fig. 1B). To generate monomeric and cyclin-complexed Cdk2 substrates in order to assay Thr^{160} phosphatase activity, we therefore first phosphorylated GST-Cdk2 with CAK before cleaving with thrombin. Half of the cleaved Thr^{160}-phosphorylated Cdk2 was incubated with buffer and the other half with purified cyclin A. Monomeric Thr^{160}-phosphorylated Cdk2 was rapidly dephosphorylated when it was added to an EDTA-treated extract from *Xenopus* eggs (Fig. 1C). In contrast, the Thr^{160} phosphate in the cyclin A–Cdk2 complex was relatively stable. Immunoblotting showed that the loss of labeling in Cdk2 was not the result of protein degradation. Similar results were obtained with a HeLa cell extract (see below). Thus, vertebrate cell extracts contain an activity that dephosphorylates Thr^{160} preferentially when Cdk2 is monomeric.

The physiological Thr^{160} phosphatase is unknown (*5*). Protein phosphatase 2A (PP2A) can dephosphorylate Thr^{160} in vitro, but this occurs in the presence or absence of cyclin (see below). The CDK-associated phosphatase KAP, a dual-specificity phosphatase (*6*) that was identified as a protein that interacts with Cdk2 and Cdc2, is a plausible candidate Thr^{160} phosphatase. KAP is related to MKP-1 and PAC1, dual-specificity phosphatases that dephosphorylate the activating threonine and tyrosine phosphates in the ERK1 and ERK2 members of the mitogen-activated protein (MAP) kinase family, which lie in the catalytic domain activation loop in a position similar to that of Thr^{160} in Cdk2 (*7*). We therefore investigated whether KAP could dephosphorylate Cdk2 Thr^{160}. KAP that was immunoprecipitated with affinity-purified antibodies to KAP (anti-KAP) from HeLa cell extracts (Fig. 2A) was found to dephosphorylate monomeric Thr^{160}-phosphorylated Cdk2, but not the cyclin A–Cdk2 complex (Fig. 2B). These results were consistent with the observed dephosphorylation of Cdk2 Thr^{160} by cell extracts (Fig. 1C). The Thr^{160} dephosphorylation activity in a HeLa cell extract was reduced after immunoprecipitation with anti-KAP (Fig. 2C), which indicates that KAP constituted a significant Thr^{160} phosphatase activity. Moreover, a bacterially expressed and purified GST-KAP protein also dephosphorylated Cdk2 Thr^{160} in the absence of cyclin A, but not in its presence (Fig. 2D). Mutation of the conserved Cys^{140} in the catalytic core of KAP (HCXXXXGR motif) (*8*) to Ser (C140S), which inactivated the phosphatase activity of KAP toward model substrates (*6*), abolished dephosphorylation of Cdk2 Thr^{160} (Fig. 2D). The dephosphorylation of Cdk2 by KAP did not depend on the kinase activity of Cdk2, because the reaction contained no Mg^{2+}–adenosine triphosphate and because a catalytically inactive mutant of Cdk2 in which Lys^{33} was changed to Arg (K33R) (*4*) was efficiently dephosphorylated (*9*). Similar results were obtained with a hexahistidine-tagged cyclin A or a protein A–tagged KAP

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(9). In contrast to KAP, purified PP2A dephosphorylated Cdk2 Thr^{160} whether or not cyclin was bound (Fig. 3A) (9).

Heat-denatured Cdk2 was not dephosphorylated by KAP (Fig. 3A), which suggests that the dephosphorylation of Cdk2 Thr^{160} by KAP requires the native Cdk2 structure, or interaction between KAP and Cdk2, or both. In contrast, PP2A nonspecifically dephosphorylated Thr^{160} in both native and denatured monomeric Cdk2 (Fig. 3A). Moreover, under the conditions used for Thr^{160} dephosphorylation of Cdk2, GST-KAP was not able to dephosphorylate Tyr^{15} in Cdk2 [the Wee1 phosphorylation site (*10*)], whereas the Tyr^{15} phosphatase GST-Cdc25B dephosphorylated Tyr^{15} in the presence or absence of cyclin (Fig. 3B).

When GST-KAP was incubated with cyclin A, Cdk2, or a cyclin A–Cdk2 complex and was then recovered with reduced glutathione (GSH)–agarose, GST-KAP associated only weakly with cyclin A but associated strongly with Cdk2 and cyclin A–Cdk2 (Fig. 4A). The ability of GST-KAP to associate with cyclin A–Cdk2 suggested that the failure of KAP to dephosphorylate cyclin A–Cdk2 was not the result of cyclin A preventing KAP from binding Cdk2. Similar binding was observed with the C140S mutant of GST-KAP, and the presence of KAP C140S did not block the phosphorylation of Thr^{160} by CAK (9).

To test whether the dephosphorylation of Cdk2 Thr^{160} by KAP inactivated Cdk2 kinase activity, we mixed Thr^{160}-phosphorylated Cdk2 with cyclin A either before or after treatment with GST-KAP (Fig. 4B). Cdk2 was inactivated only when GST-KAP was added before cyclin A, consistent with the result that GST-KAP could only dephosphorylate monomeric Cdk2. As a control, the C140S mutant of GST-KAP did not affect the kinase activity of Cdk2, irrespective of whether it was added before or after cyclin A. These data also suggest that, unlike many CDK-interacting proteins (*2*), KAP binding to cyclin A–Cdk2 did not block the kinase activity of the complex.

The overexpression of KAP slows the progression of the prereplicative G_1 phase of the cell cycle in HeLa cells (6). Could this phenomenon be caused by Cdk2 Thr^{160} dephosphorylation? When KAP was overexpressed in human 293 embryonic kidney cells by transient transfection, the amount of the faster migrating Thr^{160}-phosphorylated form (*11*) of Cdk2 was decreased (Fig. 4C). This result was not attributable to a decrease in the amount of cyclin A, Cdk7, or CAK activity (9). Given that cyclin-Cdk2 complexes are not susceptible to KAP dephosphorylation, the observed decrease in Thr^{160}-phosphorylated Cdk2 may indicate that cyclin-CDK complexes are in equilibrium in the cell and that overexpressed KAP may dephosphorylate Cdk2 when cyclin A or cyclin E dissociates. This effect of KAP could increase the length of time required for active Cdk2 complexes to accumulate to the threshold amount needed for the transition from the G_1 to the S (replication) phase and thus could account for the delay in G_1, but it is also possible

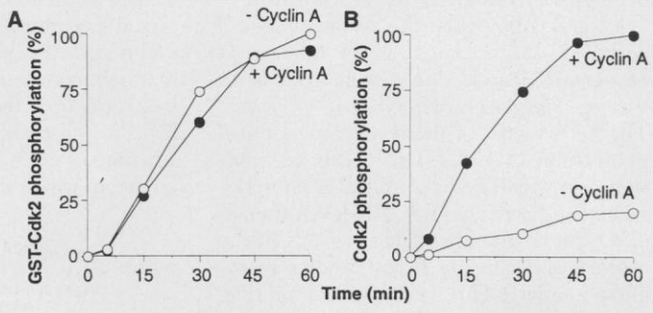

Fig. 1. CAK-phosphorylated Cdk2 as a substrate for Thr^{160} dephosphorylation. (**A**) Bacterially expressed and purified GST-Cdk2 was phosphorylated by anti-CAK immunoprecipitates in the absence (○) or presence (●) of PA–cyclin A. (**B**) Thrombin was used to cleave the GST domain from GST-Cdk2; the untagged Cdk2 was phosphorylated by anti-CAK immunoprecipitates in the absence (○) or presence (●) of PA–cyclin A. (**C**) GST-Cdk2 was phosphorylated with anti-CAK immunoprecipitates in the presence of [γ-^{32}P]ATP. Half of the GST-Cdk2 was incubated with PA–cyclin A and the other half with buffer. GST-Cdk2 was immobilized with GSH-agarose and cleaved with thrombin. The released Cdk2 was mixed with an EDTA-treated *Xenopus* egg extract, and samples were taken at the indicated times. Phosphorylation of Cdk2 is shown in the upper panel, and immunoblotting with anti-Cdk2 is shown in the lower panel; PA–cyclin A (PA-cycA) was detected by the PA domain binding to immunoglobulin G (*22*).

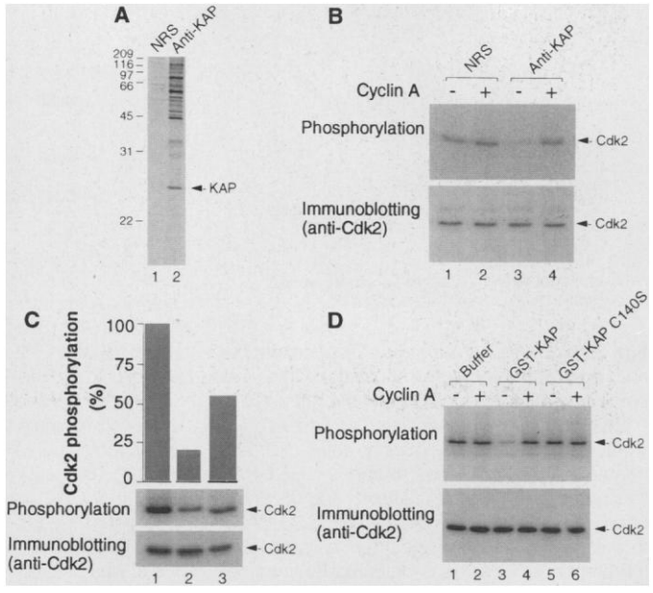

Fig. 2. (**A**) Immunoprecipitation of KAP. Cell extracts prepared from HeLa cells labeled with [^{35}S]methionine were immunoprecipitated with normal rabbit serum (NRS) (lane 1) or anti-KAP (lane 2) (*23*). The labeled proteins were detected by 17.5% SDS-PAGE followed by phosphorimaging. Molecular weight markers (in kilodaltons) are indicated at the left. (**B**) Dephosphorylation of Cdk2 Thr^{160} with anti-KAP immunoprecipitates. GST-Cdk2 was phosphorylated by CAK immunoprecipitates in the presence of [γ-^{32}P]ATP and immobilized with GSH-agarose. The phosphorylated GST-Cdk2 was cleaved with thrombin and the released Cdk2 was incubated with buffer (lanes 1 and 3) or PA–cyclin A (lanes 2 and 4). The samples were then added to an NRS immunoprecipitate (lanes 1 and 2) or an anti-KAP immunoprecipitate from HeLa cell extracts. Phosphorylation of Cdk2 was detected by SDS-PAGE followed by phosphorimaging (upper panel) and the amount of Cdk2 was detected by immunoblotting with anti-Cdk2 (lower panel) (*15*). (**C**) Dephosphorylation of Cdk2 Thr^{160} by cell extracts after immunoprecipitation with anti-KAP. Cdk2 phosphorylated on Thr^{160} with ^{32}P was incubated with buffer (lane 1), HeLa cell extracts previously immunoprecipitated with NRS (lane 2), or HeLa cell extracts previously immunoprecipitated with anti-KAP (lane 3). Phosphorylation of Cdk2 was detected by SDS-PAGE followed by phosphorimaging (middle panel) and its quantitation is shown (upper panel). The amount of Cdk2 was detected by immunoprecipitation followed by immunoblotting with anti-Cdk2 (lower panel) (*24*). (**D**) Dephosphorylation of Cdk2 Thr^{160} with recombinant KAP. GST-Cdk2 was phosphorylated, cleaved, and incubated with buffer (lanes 1, 3, and 5) or PA–cyclin A (lanes 2, 4, and 6) as in (A). The reactions were incubated with buffer (lanes 1 and 2), GST-KAP (lanes 3 and 4), or GST-KAP C140S (lanes 5 and 6) (*18*).

that KAP has other unidentified roles that contribute to the slowing of G_1.

Taken together with the observations that KAP binds to Cdc2 and Cdk2 in vivo (6), these results provide evidence that KAP dephosphorylates the activating threonine of Cdc2 and Cdk2 when the cyclin subunit is degraded or dissociates. Direct interaction between KAP and Cdk2 is likely to facilitate the specific dephosphorylation of Cdk2 Thr^{160}. A model of the regulation of phosphorylation of Cdk2 Thr^{160} can be envisioned in which cyclin A–Cdk2 is phosphorylated and activated by CAK [even though Cdk2 can be phosphorylated by CAK in the absence of cyclin (*3*, *4*) and binding to cyclin potentiates Thr^{160} phosphorylation (Fig. 1B)]. KAP can bind to Thr^{160}-phosphorylated cyclin A–Cdk2 and does not block its kinase activity, but Thr^{160} is not accessible for dephosphorylation by KAP. When cyclin A is degraded, the phosphorylated Thr^{160} becomes accessible to KAP and is dephosphorylated. Hence, cyclin A may induce Cdk2 Thr^{160} phosphorylation both by stimulating CAK phosphorylation and by inhibiting dephosphorylation by KAP. Thr^{160} is located in the flexible activation loop in the crystal structure of Cdk2 (*12*); the binding of cyclin and the phosphorylation of Thr^{160} may both contribute to the movement of the loop away from the catalytic core and possibly away from KAP. On the basis of the structure of cyclic AMP–dependent protein kinase, in which the activating phosphate at Thr^{197} is anchored by multiple interactions and is thereby shielded from phosphatase action, the phosphate on Thr^{160} may also be partly masked in the active cyclin-bound state and may thus be inaccessible to KAP (although it is still accessible to PP2A). In addition, cyclin A might interact with KAP directly (Fig. 3C) and pull it away from Thr^{160}.

Although monomeric Thr^{160}-phosphorylated Cdk2 is not active as a kinase, dephosphorylation of the monomeric Cdk2 Thr^{160} in the cell may be essential to ensure that Cdk2 is not immediately activated when cyclins are synthesized in the next cycle. KAP interacts with Cdc2, Cdk2, and Cdk3 but not with Cdk4 or Cdk5 (6); hence, it is likely that KAP can also dephosphorylate the equivalent threonine in Cdc2 and Cdk3. Like MKP-1 and PAC1, KAP can inactivate a specific target protein kinase by removing phosphates in the activation loop, and this ability may constitute a general role for members of the dual-specificity phosphatase family.

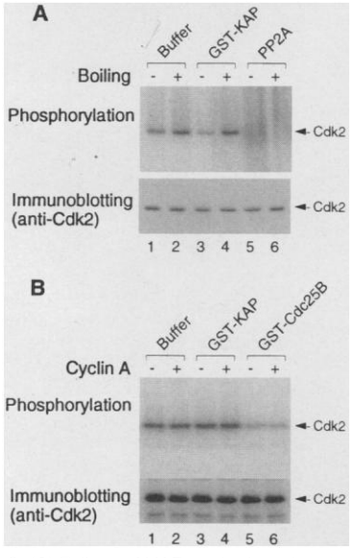

Fig. 3. (**A**) Failure of KAP to dephosphorylate denatured Cdk2. Thr^{160}-phosphorylated Cdk2 was prepared as above and boiled in the presence of 0.5% SDS for 5 min (lanes 2 and 4). The samples were immunoprecipitated with anti-Cdk2 and treated with buffer (lanes 1 and 2), GST-KAP (lanes 3 and 4), or PP2A (lanes 5 and 6). Phosphorylation of Cdk2 was detected by SDS-PAGE followed by phosphorimaging (upper panel) and the amount of Cdk2 was detected by immunoblotting with anti-Cdk2 (lower panel) (*25*). (**B**) Failure of KAP to dephosphorylate Tyr^{15}. GST-Cdk2 was phosphorylated by GST-Wee1 in the presence of [γ-^{32}P]ATP. The GST-Cdk2 was recovered with GSH-agarose and cleaved with thrombin. The cleaved Cdk2 was incubated with buffer (lanes 1, 3, and 5) or PA–cyclin A (lanes 2, 4, and 6). The samples were then incubated with buffer (lanes 1 and 2), GST-KAP (lanes 3 and 4), or GST-Cdc25B (lanes 5 and 6). Phosphorylation of Cdk2 was detected by SDS-PAGE followed by phosphorimaging (upper panel) and the amount of Cdk2 was detected by immunoblotting with anti-Cdk2 (lower panel) (*26*).

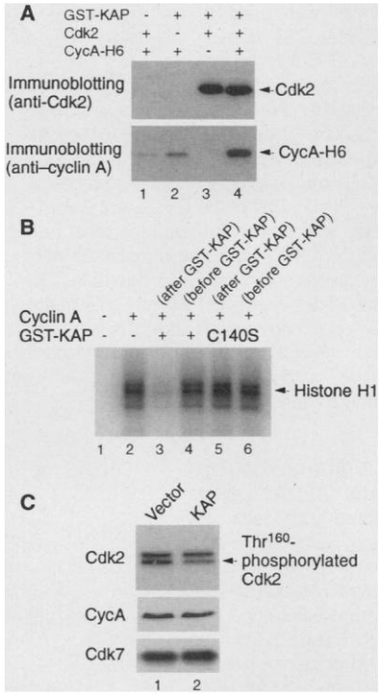

Fig. 4. (**A**) Association of KAP with Cdk2 and cyclin A. GST-Cdk2 was immobilized with GSH-agarose and cleaved with thrombin. The released Cdk2 (lanes 1, 3, and 4) was incubated with cyclin A–H6 (CycA-H6) (lanes 1, 2, and 4) and GST-KAP (lanes 2 to 4). The GST-KAP was recovered with GSH-agarose and the associated Cdk2 (upper panel) and cyclin A (lower panel) were detected by immunoblotting with the corresponding antibodies (*27*). (**B**) Inactivation of the histone H1 kinase activity of Cdk2 by KAP. GST-Cdk2 was phosphorylated by anti-CAK immunoprecipitates and then immobilized with GSH-agarose. The GST-Cdk2 was cleaved with thrombin and the released Cdk2 was incubated with GST-KAP (lanes 3 and 4) or GST-KAP C140S (lanes 5 and 6). CycA-H6 (lanes 2 to 6) was incubated with Cdk2 either before (lanes 4 and 6) or after (lanes 3 and 5) incubation with GST-KAP. The histone H1 kinase activity of the samples was then assayed (*28*). (**C**) Reduction of Cdk2 Thr^{160} phosphorylation by overexpression of KAP. Vector alone (lane 1) or KAP-expressing plasmid (lane 2) were transfected into human 293 embryonic kidney cells. Cell extracts were prepared and resolved on a long SDS-polyacrylamide gel followed by immunoblotting with anti-Cdk2 (upper panel), anti–cyclin A (middle panel), and anti-Cdk7 (lower panel) (*29*).

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15. GST-Cdk2 (10 μg) was phosphorylated by anti-CAK immunoprecipitates as described (*13*). Phosphorylated GST-Cdk2 was immobilized on GSH-agarose and cleaved with thrombin as described (*4*). The thrombin was inhibited by 1 U of hirudin (Sigma). Cdk2 (100 ng) in the supernatant was incubated with buffer or protein A (PA)–cyclin A (100 ng) at 4°C for 15 min; the Cdk2 or PA–cyclin A–Cdk2 was mixed with an anti-KAP immunoprecipitate (from 200 μg of HeLa cell extracts made with 1 μg of anti-KAP) in buffer (containing 20 mM tris-Cl (pH 7.5), 50 mM NaCl, and 0.1 mM EDTA) and incubated at 23°C for 45 min.
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18. Human KAP complementary DNA (cDNA) was obtained by the polymerase chain reaction (PCR) from HeLa cell cDNA (provided by W. Jiang) with the oligonucleotides 5′-GCCAGCCATGGAGCCGCCCAGTTCAAT-3′ and 5′-TGAATTCTCGAGTCTTGATACAGATCTTGA-3′.

The PCR product was digested with Nco I and Xho I and cloned into pGEX-KG vector (*16*). GST-KAP C140S was constructed by a PCR method as described (*17*) with the oligonucleotide 5′-TTAATACACAGCTATGGAGG-3′ and its antisense. Phosphorylated Cdk2 was prepared as in (*15*) and incubated with 100 μg of GST-KAP or GST-KAP C140S in buffer containing 20 mM tris-Cl (pH 7.5), 50 mM NaCl, and 0.1 mM EDTA at 23°C for 45 min.

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22. Expression and purification of human GST-Cdk2 and bovine PA–cyclin A (staphylococcal PA-tagged) was as described (*4*). Phosphorylation of GST-Cdk2 or Cdk2 by anti-CAK immunoprecipitates (from mouse Swiss 3T3 cells) in the presence of [γ-^{32}P]ATP was as described (*13*); phosphorylation was quantitated with a phosphorimager (Molecular Dynamics). GST-Cdk2 was immobilized on GSH-agarose and cleaved with thrombin as described (*4*); the cleaved Cdk2 in the supernatant was incubated with PA–cyclin A (100 ng) at 4°C for 15 min. Phosphorylated Cdk2 (100 ng in 1 μl) was added to *Xenopus* cytostatic factor–arrested egg extract (10 μl) treated with 5 mM EDTA and incubated at 23°C. Anti-Cdk2 and immunoblotting were as described (*13*).
23. HeLa cells were labeled with [^{35}S]methionine for 6 hours as described (*14*). Cell extracts were prepared (*13*), and 500 μg was immunoprecipitated (*13*) with 1 μg of affinity-purified anti-KAP directed against the COOH-terminal 18 residues of KAP (C-18; Santa Cruz Biotechnology, Santa Cruz, CA).
24. Phosphorylated Cdk2 (100 ng) (*15*) was added to a HeLa cell extract (200 μg) that had been immunoprecipitated with anti-KAP as described (*15*) and treated with 5 mM EDTA. After incubation at 23°C for 30 min, the Cdk2 was recovered by immunoprecipitation with anti-Cdk2 (*13*).
25. PP2A was purified from bovine brain (provided by K. P. Lu) and was used at a concentration of 0.24 U/ml. The Cdk2 Thr^{160} dephosphorylation assay was as described (*18*).
26. Human *Wee1Hu* cDNA (*19*) cloned into pGEX-KG was provided by K. Yamashita. Mouse GST-Cdc25B was as described (*20*). Monoclonal antibody to cyclin A was provided by J. Gannon. Phosphorylation of GST-Cdk2 Tyr^{15} was done by incubating GST-Cdk2 (10 μg) with GST-Wee1 (1 μg), 15 mM $Mg(OAc)_2$, and 10 μCi of [γ-^{32}P]ATP in a volume of 10 μl at 23°C for 60 min. The GST fusion proteins were recovered with GSH-agarose, cleaved with thrombin, and treated with hirudin; the cleaved Cdk2 (100 ng) in the supernatant was incubated with 100 ng of GST-KAP or GST-Cdc25B as described (*18*).
27. Cyclin A–H6 is a histidine-tagged truncated bovine cyclin A that can bind Cdk2 (R. Y. C. Poon and T. Hunt, unpublished data) and was expressed in bacteria and purified as described (*21*). GST-Cdk2 was cleaved with thrombin and treated with hirudin as above. The cleaved Cdk2 (100 ng) was incubated with cyclin A–H6 (100 ng) and GST-KAP (1 μg) with bovine serum albumin (5 μg) as a carrier at 23°C for 30 min; the GST-KAP was then recovered with GSH-agarose.
28. GST-Cdk2 was phosphorylated with CAK immunoprecipitates in the presence of 1 mM ATP and 15 mM Mg^{2+}; the phosphorylated GST-Cdk2 was immobilized on GSH-agarose, cleaved with thrombin, and treated with hirudin as above. The cleaved Cdk2 (100 ng) was incubated with cyclin A–H6 (1 μg) and then with GST-KAP or C140S mutant (1 μg), followed by cyclin A–H6 (1 μg) as indicated; each incubation was at 23°C for 15 min. The histone H1 kinase assay was as described (*4*).
29. KAP was subcloned into pcDNA3 vector (Invitrogen) from the Bam HI–Xho I fragment of GST-KAP in pGEX-KG. KAP in pcDNA3 or pcDNA3 vector (20 μg) was transfected into human 293 embryonic kidney cells (5 × 10^5 cells per 10-cm plate) by a calcium phosphate precipitation method. Cell extracts were prepared and resolved by SDS–polyacrylamide gel electrophoresis (PAGE) (15%, 15 cm in length) followed by immunoblotting with anti-Cdk2, anti–cyclin A (*4*), and anti-Cdk7 (provided by E. Nigg).
30. We thank T. Hunt, in whose laboratory this work was first conceived. Supported by NIH grants CA14195 and CA39780 to T.H. R.Y.C.P. is a fellow of the International Human Frontier Science Program. T.H. is an American Cancer Society Research Professor.

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Prion-Inducing Domain of Yeast Ure2p and Protease Resistance of Ure2p in Prion-Containing Cells

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The genetic properties of the [URE3] non-Mendelian element of *Saccharomyces cerevisiae* suggest that it is a prion (infectious protein) form of Ure2p, a regulator of nitrogen catabolism. In extracts from [URE3] strains, Ure2p was partially resistant to proteinase K compared with Ure2p from wild-type extracts. Overexpression of Ure2p in wild-type strains induced a 20- to 200-fold increase in the frequency with which [URE3] arose. Overexpression of just the amino-terminal 65 residues of Ure2p increased the frequency of [URE3] induction 6000-fold. Without this "prion-inducing domain" the carboxyl-terminal domain performed the nitrogen regulation function of Ure2p, but could not be changed to the [URE3] prion state. Thus, this domain induced the prion state in trans, whereas in cis it conferred susceptibility of the adjoining nitrogen regulatory domain to prion infections.

A prion is an infectious protein, an altered form of a normal protein that may have lost its normal function but has acquired the ability to convert the normal form into the altered (prion) form. This concept originated in studies of scrapie of sheep, kuru and Creutzfeldt-Jakob diseases of humans, and bovine spongiform encephalopathy (mad cow disease) (*1*, *2*). These diseases are believed to be caused by a self-propagating conformational change of a highly conserved protein denoted PrP.

We previously suggested that [URE3] and [PSI], two non-Mendelian elements of yeast, are prion forms of the chromosomally encoded Ure2p and Sup35p, respectively (*3*). [URE3] (*4*, *5*) or mutations in the chromosomal *URE2* gene (*6*) each produce derepression of nitrogen catabolic enzymes that would normally be repressed by a good nitrogen source (*7*). [PSI] (*8*, *9*) or mutations in *SUP35* (*10*) both increase the efficiency of nonsense suppressor tRNAs in yeast. We proposed that [PSI] and [URE3] are prions on the basis of three lines of evidence.

1) Each is reversibly curable. Curing of [PSI] by high osmotic strength (*11*) or guanidine (*12*) produces colonies from which [PSI]-containing clones can again be derived, at some low frequency (*12*, *13*). Likewise, curing of [URE3] by 5 mM guanidine produces strains from which [URE3] clones can again be isolated (*3*). This is unlike nucleic acid replicons which, once cured, do not return unless introduced from other cells. Guanidine curing may be mediated by induction of Hsp104 (*14*).

2) [PSI] and [URE3] depend for their propagation on the chromosomal *SUP35* and *URE2* genes, respectively (*3*, *5*, *15*, *16*). The phenotypes of recessive *sup35* and *ure2* mutants closely resemble those produced by the presence of [PSI] and [URE3], respectively. In contrast, recessive mutants unable to propagate nucleic acid replicons have phenotypes opposite to those produced by the presence of these genomes.

3) Overproduction of Ure2p induces the generation of [URE3] (*3*) and overproduction of Sup35p induces the generation of [PSI] (*13*). The presence of more of the normal form increases the likelihood that the spontaneous prion change will occur.

The relative protease resistance of PrP from diseased animals compared with that from normal animals was an early indication that the mammalian scrapie agent was an altered form of this protein (*17*). It has also been critical in the recent demonstration of in vitro conversion of PrP^{C} to PrP^{Sc} (*2*). By immunoblotting with a polyclonal antibody to Ure2p (*3*), we detected equal amounts of similarly migrating Ure2p in extracts of strains with and without [URE3] (Fig. 1A, lanes marked X). Ure2p in extracts of normal strains was digested by proteinase K in less than 1 min to products that run off the gel, whereas Ure2p from isogenic [URE3] (prion-con-

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